Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in *EGFR* in circulating lung-cancer cells. N Engl J Med 2008;359:366-77. DOI: 10.1056/NEJMoa0800668.

SUPPLEMENTAL TEXT

Additional Methods (online only)

In addition to the information in the main paper, the following methods were utilized.

CTC Analysis

For CTC capture, whole blood was passed through the CTC-chip at a flow rate of 1-2ml/hr allowing capture of CTCs, followed by saline wash (10ml/hr) to remove non-specifically bound leukocytes. For CTC enumeration, cells were fixed on the CTC-chip (1% paraformaldehyde, 0.2% Triton X-100, 1% bovine serum albumin (BSA), all in phosphate buffered saline (PBS)) and stained with Hoechst 33342 to identify DNA content, phycoerythrin -conjugated antibody to cytokeratin (CK) to identify epithelial cells and fluorescein-conjugated antibody to CD45 to identify leukocytes. The number of CTCs/ml was determined via comprehensive image analysis, scanning the entire chip (Olympus SZX microscope,Olympus America Inc., NY) and identifying CTCs based on cell size, morphology and fluorescence staining (Hoechst, CK positive). For demonstration of EGFR expression, captured cells were stained with a mouse monoclonal antibody (Vector Laboratories).

Clinical cohorts and analysis

Progression-free survival (the time from start of EGFR TKI therapy until tumor progression or death) according to T790M mutation status among patients with activating *EGFR* mutations was analyzed with a multi-variable Cox model controlling for both gender and presence of brain metastases. Since all patients had excellent performance

status, adenocarcinoma histology, and were never or light smokers, these prognostic factors were not included in the model. As per IRB protocol, the acquisition of blood samples for CTC analysis was dependent upon previously scheduled clinical encounters. Thus, not all CTC samples were available for detailed longitudinal genotyping analyses.

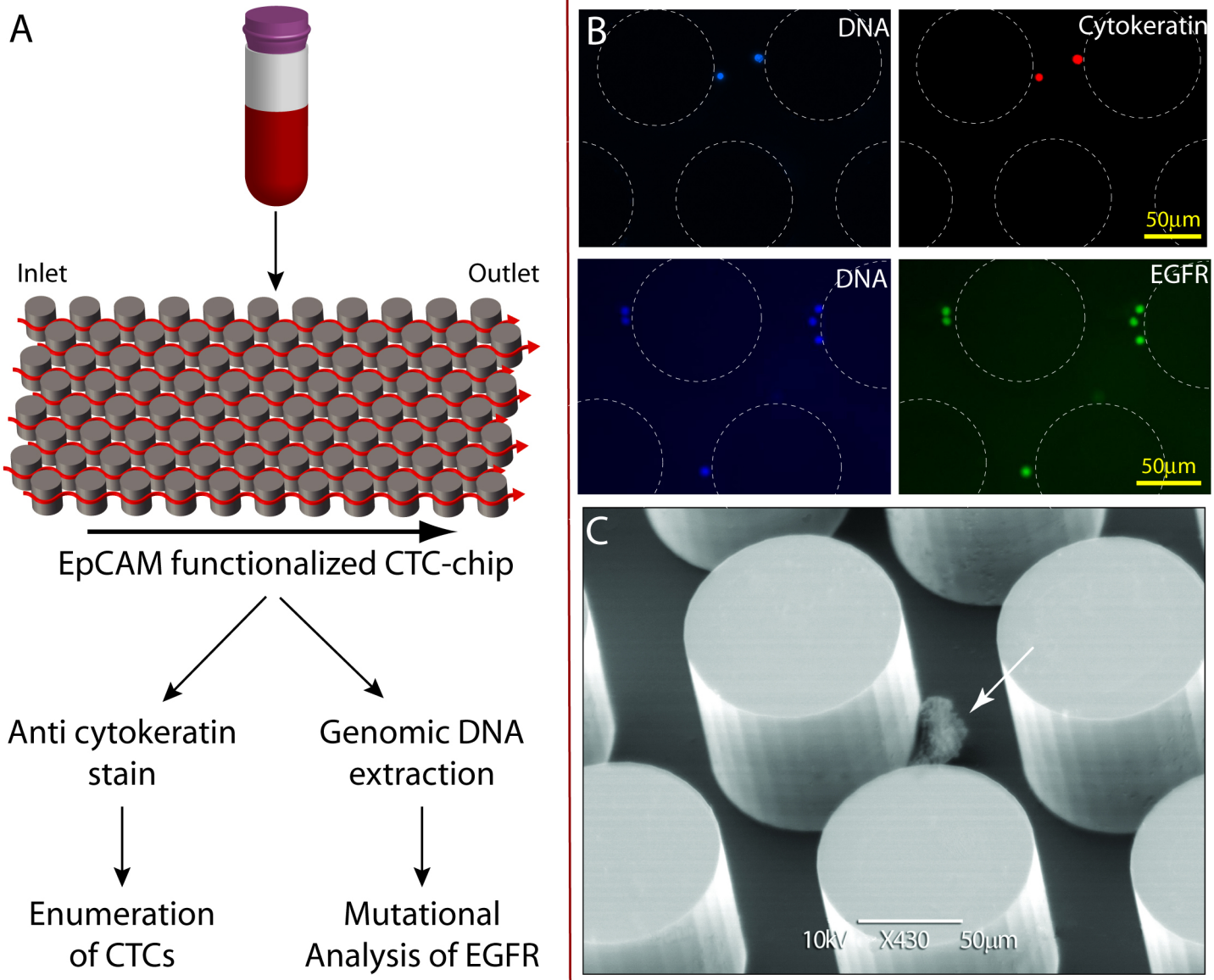
Responsibilities of authors:

All authors made significant contributions to either the design, data collection and analysis, and writing of the manuscript. The three joint first authors (SM, LVS, SN) and the two senior authors (MT and DAH) were responsible for planning the experimental studies. Clinical samples and data analyses were undertaken by LVS, SD, AM, TJL; CTC purification was performed by SN, LU, BB, CC, EI; molecular studies were performed by SM, LU, BB, SD, AJI, DWB; and results were analyzed and discussed by SM, LVS, SN, DI, JS, RGT, TJL, MT and DAH. The first draft of the manuscript was written by SM. The research was supported by NIH grants and unrestricted donations from philanthropic foundations as listed under Acknowledgements. No commercial funding was obtained to support this study, and it was not subject to any confidentiality agreements.

Supplementary Table1: Longitudinal analysis of activating *EGFR* genotypes in circulating tumor cells from patients with non small cell lung cancer

Patient Number	**1**	**2**	**3**	**10**	**20**	**22**	**23**
Mutation at Diagnosis[1]	**Del**	**Del[2]**	**G719X**	**L858R**	**S885L[3]**	**Del**	**Del**
Time of CTC analysis[4]							
<100d		**Del**			**Del**	**Del G719X**	**Del L858R**
101-150d	**Del**			**L858R Del**			
151-200d	**Del**			**G719X**			
201-250d	**Del L858R**						
251-300d		**Del**	**G719X Del**	**Del**			
301-350d		**Del, L858R L861Q**	**G719X Del**				
351-400d		**Del**		**Del**			

[1]The EGFR mutation at diagnosis was identified by nucleotide sequencing of tumor biopsies at the time of presentation. Serial CTC genotypes, treatment course, CTC quantification and tumor volume measurements for cases 1 and 10 are depicted graphically in figure 2A. Direct *EGFR* nucleotide sequencing of case 2 is shown in Figure 3: [2]The specific deletion mutation identified in the primary tumor differs from that observed in followup CTC specimens. [3]The S885L mutation identified in the tumor specimen is of unknown significance and is not represented in the SARMS assay. No deletion mutation was identified in the tumor. [4]The timing of repeated CTC genotyping analysis following the initial sample is shown in days (d).


A
Inlet
Outlet
EpCAM functionalized CTC-chip
Anti cytokeratin stain
Genomic DNA extraction
Enumeration of CTCs
Mutational Analysis of EGFR
B
DNA
Cytokeratin
50µm
DNA
EGFR
50µm
C
10kV X430 50µm

Supplementary figure 1: CTC-chip analysis of blood specimens from NSCLC patients

A. Schematic representation of CTC-chip analysis. Whole blood is collected from the patient and passed through the CTC-chip, containing 78,000 microposts coated with antibody to the epithelial surface antigen EpCAM. For CTC enumeration, captured cells are stained *in situ* using an antibody to cytokeratin; for molecular studies, captured cells are lysed on the chip and eluted DNA undergoes the desired analysis.

B. Visualization of CTCs captured against the sides of the functionalized microposts (dashed lines superimposed on images). DNA staining is used to identify all nucleated cells within a field. Cells here are also stained with rhodamine-conjugated antibody to cytokeratin (red) or fluorescein-conjugated antibody to EGFR (green); magnification 200X

C. Scanning electron microscopic image of a single CTC captured from a patient with NSCLC (arrow).